

Indu J. Isaacs

	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430	2431	2432	2
--	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	---

GLP-2 FORMULATIONS

FIELD OF INVENTION

The present invention provides formulations for GLP-2 peptides and analogs thereof.

5 In particular, the invention provides formulations of GLP-2 peptides and GLP-2 analogs with improved stability.

BACKGROUND OF THE INVENTION

Administration of therapeutic peptides requires peptide formulations that remain stable during storage. In general, parenteral administration is used with peptides because of their increased size and subsequent difficulty in crossing biological membranes. Peptides can be particularly difficult to formulate because of their tendency to degrade over time and/or undergo aggregation and precipitation. Degradation, aggregation, and precipitation are all indicative of an unstable formulation. Such an unstable formulation is not commercially viable, as it cannot pass U.S. Food and Drug Administration approval.

Formulation variables which affect the degradation of peptides during storage include, but are not limited to, pH, the quantity of salts present, and the type and quantity of excipients. In addition, temperatures, pressures, and time for freezing and drying cycles can affect the stability of a lyophilized peptide formulation. The role of most of these variables has been studied; however, the synergistic effect of the variables is still poorly understood.

20 Glucagon-like peptide-2 (GLP-2) is a 33 amino acid peptide having therapeutic applications in the treatment of diseases of the gastrointestinal tract. In particular, it has been determined that GLP-2 and analogs thereof act as trophic agents to enhance and maintain the functioning of the gastrointestinal tract and to promote growth of intestinal tissue. *See e.g.*, U.S. Patent Nos. 5,834,428; 5,789,379; and 5,990,077; and International Publication No. WO 98/52600.

25 Commercial exploitation of GLP-2 or an analog thereof requires a stable GLP-2 formulation that can be readily prepared using a commercially acceptable process. Because GLP-2 is a protein, and thus far more labile than traditional small molecular weight drugs, the formulation of GLP-2 or an analog thereof presents challenges not commonly encountered by the pharmaceutical industry. For example, methionine oxidation at position 10 and asparagine deamination at position 11, 16, and/or 24 of GLP-2 are potential routes of degradation.

Furthermore, GLP-2 or an analog thereof may also be adsorbed to surfaces to form aggregates and/or precipitate, which would then render the formulation unstable.

There is a need in the art for stable formulations of GLP-2 peptides and analogs thereof which can be prepared using a commercially acceptable process. The present invention satisfies these needs.

SUMMARY OF THE INVENTION

The present invention provides stable formulations of GLP-2 and analogs thereof, which can be prepared using a commercially acceptable process.

It has been discovered that relatively high concentrations of GLP-2 can be used in pharmaceutically acceptable formulations. Moreover, it has been discovered that a pH of greater than about 5.5, more preferably greater than about 6, even more preferably from about 6.9 to about 7.9, and most preferably about 7.3 to about 7.4, is suitable for a stable formulation.

It has also been discovered that the GLP-2 analog h[Gly2]GLP-2 undergoes a phase transition between 40-55°C, depending upon the salt concentration, and becomes hydrophobic in the presence of salt. It has also been discovered that Tween 80®, salt, and arginine are not suitable materials for producing a stable formulation for h[Gly2]GLP-2.

According to one aspect of the present invention, there is provided a GLP-2 formulation comprising: (1) a medically useful amount of GLP-2; (2) a phosphate buffer sufficient to adjust the pH of the formulation to a pharmaceutically acceptable level, and in particular above about 6.0; (3) a stabilizing amount of the amino acid L-histidine; and (4) a bulking agent selected from sucrose and mannitol.

More particularly, there is provided a GLP-2 formulation comprising: (1) a medically useful amount of GLP-2 comprising from about 0.1 to about 50 mg/ml of GLP-2, preferably about 5 to about 40 mg/ml, more preferably about 7 to about 30 mg/ml, even more preferably about 10 to about 20 mg/ml, and most preferably about 20 mg/ml; (2) a phosphate buffer to maintain the pH at a physiologically tolerable level, i.e., above 6; (3) a stabilizing amino acid, particularly L-Histidine; and (4) a bulking agent, particularly mannitol. All percentages described herein (except for percentages for water) are weight/volume of formulated product prior to lyophilization in gms/ml (x100). Percentages for water content are weight/weight of lyophilized product (x100).

In one embodiment of the present invention, the GLP-2 formulation is a h[Gly2]GLP-2 lyophilized formulation comprising in the reconstituted product: (1) phosphate buffer in an amount necessary to maintain the pH of the reconstituted product between about 6.9-7.9, and preferably in an amount to maintain a pH of about 7.3 to about 7.4; (2) about 0.5 to about 1% L-histidine; (3) about 2 to about 5% mannitol, preferably about 2.5 to about 3.5% mannitol, and most preferably about 3% mannitol; and (4) from about 0.1 to about 50 mg/ml of GLP-2 or an analog thereof, preferably about 5 to about 40 mg/ml, more preferably about 7 to about 30 mg/ml, even more preferably about 10 to about 20 mg/ml, and most preferably about 20 mg/ml.

In a more preferred embodiment of the invention, a h[Gly2]GLP-2 lyophilized formulation is provided comprising in the reconstituted product: (1) about 7 to about 30 mg/ml, preferably about 10 to about 20 mg/ml, and most preferably about 20 mg/ml of h[Gly2]GLP-2; (2) a phosphate buffer sufficient to maintain the pH at about 7.3 to about 7.4; (3) about 0.5 to about 1% L-histidine; and (4) about 3% mannitol.

In another aspect of the present invention there is provided a process for making the lyophilized formulation of GLP-2. Such a process comprises the following steps:

- (a) preparing the GLP-2 formulation comprising GLP-2 or an analog thereof, a phosphate buffer, L-histidine, and mannitol;
- (b) freezing the formulation to about -40°C ;
- (c) performing a first drying step at about -20°C ; and
- (d) performing a second drying step at $+20^{\circ}\text{C}$.

In a preferred embodiment the liquid formulation subjected to the lyophilization process comprises:

- (1) the h[Gly2]GLP-2 analog; (2) 35 mM phosphate buffer to maintain the reconstituted product at a pH of about 6.9 to about 7.9, and more preferably at a pH of about 7.3 to about 7.4; (3) about 0.5 to about 1% L-histidine; and (4) about 3% mannitol.

According to another aspect of the present invention, there is provided a method for preparing a GLP-2 pharmaceutically acceptable formulation for parenteral administration, comprising the step of reconstituting the lyophilized GLP-2 formulation.

There is further provided in accordance with the present invention a therapeutically useful kit comprising: (1) a sterile vial comprising a lyophilized GLP-2 formulation of the invention, (2) a vehicle suitable for reconstitution thereof, preferably sterile water, (3) instructions for reconstitution; and (4) optionally instructions for administration. The kit may further comprise a device suitable for injection of the reconstituted preparation.

Both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

5

Brief Description of the Figures

Figure 1: Shows a bar graph of the effect of certain amino acid stabilizers on a formulation of h[Gly2]GLP-2 using a heat stress test. The percent (%) purity is plotted for three different amino acid formulations, both before and after the application of heat;

10

Figure 2: Shows a bar graph of the effect of L-histidine on a phosphate buffered formulation of h[Gly2]GLP-2. The % purity is plotted for three different formulations at 0 and at 4 hours;

Figure 3: Shows a bar graph of the screening of bulking agents analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) at room temperature and 60°C. The % purity is plotted for seven different amino acid formulations;

Figure 4: Shows a bar graph of the screening of bulking agents analyzed by size exclusion high performance liquid chromatography (SE-HPLC). "HMW" represents a high molecular weight peak. The % purity is plotted for seven different formulations;

Figure 5: Shows a bar graph of the stability of mannitol and sucrose formulations of h[Gly2]GLP-2 in a liquid state, prior to lyophilization, which have been stored at 4°C. The % purity is plotted for four different formulations at 0 min. through 49 min., at 7 min. intervals; and

Figure 6: Shows a bar graph of the stability of lyophilized mannitol and sucrose formulations of h[Gly2]GLP-2 which have been stored at 60°C. The % purity is plotted for four different amino acid formulations.

30

Detailed Description of the Invention

The invention relates to GLP-2 formulations which exhibit superior storage stability. The term "GLP-2," as used herein, means a naturally occurring GLP-2 peptide or a GLP-2 analog thereof (unless specifically indicated otherwise).

5 The present GLP-2 formulations can be provided as liquid formulations suitable for administration, such as by injection, in unit or multi-dose amounts. The liquid formulations can also serve as stock solution from which lyophilized dosage forms can be prepared. Accordingly, the present GLP-2 formulations can also be provided in lyophilized form, e.g., as freeze-dried powders suitable for reconstitution and subsequent administration as
10 injectable liquid formulations.

Lyophilized formulations of the present invention exhibit storage stability of six months at ambient temperature, and eighteen months at 4°C. Storage stability is exhibited by minimal peptide degradation, preferably less than about 5% peptide degradation, more preferably less than about 3 to about 4% peptide degradation, and even more preferably less than about 1 to about 2% peptide degradation. Peptide degradation can be measured using
15 standard reverse-phase HPLC (RP-HPLC) techniques.

The naturally occurring GLP-2 peptides are highly conserved peptides. Accordingly, GLP-2 peptides for use in the present invention include the various naturally produced forms of GLP-2, particularly vertebrate species (including piscine and avian species), more particularly mammalian (such as primate, rodent (including rat, mouse, degu, hamster, and
20 guinea pig), porcine, and bovine,), and more particularly the human form. Desirably, but not essentially, the naturally occurring GLP-2 peptide selected for use is of the same species as the subject identified for treatment.

GLP-2 analogs potentially useful in the present invention include agonists and
25 antagonists of the GLP-2 receptor. GLP-2 agonists activate the GLP-2 receptor by first binding to the receptor, followed by stimulating an intracellular second messenger system coupled to the receptor. In one embodiment of the invention, the GLP-2 agonists act selectively at the GLP-2 receptor. Selectively-acting GLP-2 agonists are compounds that, in the context of a suitable GLP-2 receptor binding or functional assay, bind to the GLP-2
30 receptor with greater affinity. Such greater affinity is preferably at least an order of magnitude greater relative to different receptor types, such as the GLP-1 receptor. In other embodiments, the GLP-2 analogs bind to the GLP-2 receptor with an affinity at least equivalent to the affinity of naturally occurring GLP-2.

In other embodiments of the invention, the GLP-2 peptide is an analog of natural GLP-2 that incorporates one or more amino acid substitutions, additions, deletions, or modifications and retains biological activity.

The agonist activity of human GLP-2 and rat GLP-2 is believed to require an intact N-terminus, but various deletions of up to several residues at the C-terminus are tolerated without loss of agonist activity. Substitutions are tolerated at sites outside regions conserved across the various GLP-2 species homologs. Similarly, substitutions are also tolerated at sites within regions conserved across GLP-2 species. In preferred embodiments, the amino acid substitutions are conservative substitutions. For example, one member of an amino acid class can be substituted by another member, *e.g.*, the substitution of alanine by glycine, the substitution of asparagine by glutamine, the substitution of methionine by leucine or isoleucine, and the like.

Antagonist activity of GLP-2 analogs in humans and rats is exhibited when the naturally occurring GLP-2 peptide is mutated in any one or more of the first four N-terminal residues, in particular by deleting any one or more of these N-terminal residues. In addition, antagonist activity is exhibited when naturally occurring hGLP-2 is substituted: (1) with an amino acid which does not naturally occur at any of the following positions: Asp¹⁵, Phe²², Thr²⁹, Thr³² and/or Asp³³; (2) and when Ala² is replaced by anyone of the following amino acids: Leu, Cys, Glu, Arg, Trp and PO₃-Tyr². In addition, antagonists of GLP-2 analogs include any mutation or variation of the naturally occurring GLP-2 peptide which results in the inhibition of intestinotrophic activity of naturally occurring GLP-2 or GLP-2 analogs which exhibit agonist activity. Structural analogs of GLP-2 which act as antagonists are specifically described in WO 98/03547.

The GLP-2 receptor analogs can be identified by screening peptides against cells genetically engineered to produce the GLP-2 receptor. The GLP-2 receptor has been cloned. *See* Munroe et. al., *Proc. Natl. Acad. Sci. USA*, 96(4):1569 (1999). Cells functionally incorporating the GLP-2 receptor, and their use to screen GLP-2 analogs, are also described in International Publication No. WO 98/25955, published on June 18, 1998.

In a preferred embodiment, the GLP-2 analog with agonist activity has been altered to confer resistance to degradation by endogenous enzymes, such as DPP-IV. Such analogs suitably incorporate a replacement of the alanine residue at position 2. In specific embodiments, the Ala² residue is replaced by glycine or serine, or by other residues as described for example in U.S. Patent No. 5,789,379. In a preferred embodiment, the GLP-2 receptor agonist is [Gly²]GLP-2. For use in treating humans, the GLP-2 analog is desirably

but not essentially a human GLP-2 peptide or analog, particularly including the Gly2 analog of human GLP-2.

It was discovered that the h[Gly2]GLP-2 analog precipitated at a pH of less than 5.5, and that temperature profiles suggested a heat-induced and salt-dependent transition temperature of about 40°C. Based on pH solubility profiles, it was determined that a phosphate buffer provides optimal buffering capacity for GLP-2 peptides. Furthermore, the addition of L-histidine to the phosphate buffer was found to effectively stabilize GLP-2 peptides, whereas the addition of arginine citrate or lysine did not effectively stabilize GLP-2 compositions. L-histidine acts as a stabilizing amino acid that increases the length of time that the GLP-2 peptide remains intact prior to degradation.

The lyophilized formulations of the present invention are preferably provided in a powder form comprising not more than about 5% water by weight, preferably not more than 2% water by weight, and more preferably not more than about 1% water by weight.

The bulking agent incorporated in the preparation produces a non-crystalline amorphous cake. It was found that lactose, trehalose, and maltose sugars did not effectively stabilize the GLP-2 formulation as well as mannitol and sucrose. Mannitol was found to be the preferred excipient for the GLP-2 formulations.

The buffering agent incorporated in the formulation of the present invention is selected from those capable of buffering the preparation to a pH within a physiologically tolerable range for administration to a patient. "Physiologically tolerable" formulations are those that elicit reactions, in a recipient, that are not so extreme as to preclude further administration of the formulation. acceptable range for administration to a patient. More particularly, it was found that the pH of the formulation should be greater than about 5.5, more preferably greater than about 6, even more preferably of about 6.9 to about 7.9, and most preferably about 7.3 to about 7.4. Preferably, the buffering agent is phosphate based, and most preferably a 35 mM phosphate buffer is used.

The formulations of the present invention incorporate GLP-2 in a medically effective amount, namely an amount which is useful either therapeutically or diagnostically. Such an amount can be determined based on the type of GLP-2 peptide or analog selected and on the intended end-use of the preparation. Therapeutically useful amounts of GLP-2 include those unit dosage amounts useful in a regimen to treat a subject that would benefit from GLP-2 administration, as described more fully in U.S. Patent Nos. 5,834,428; 5,789,379; 5,990,077; and 5,952,301, and in International Publication No. WO 98/52600.

In one application, the formulation may be exploited for the treatment of gastrointestinal disease, particularly diseases, disorders or conditions of the intestine. Therapeutically useful amounts also include multi-dose amounts of GLP-2, which can be delivered to an intended subject. Diagnostically useful amounts of GLP-2 include those amounts useful as a calibrant when assessing endogenous levels of GLP-2 or levels of GLP-2 drug in a subject, for instance as a prelude to GLP-2 therapy, or during the course of GLP-2 treatment. Medically useful amounts of GLP-2 thus can range widely from a few micrograms to many milligrams. The formulations of the present invention preferably provide about 0.1 to about 50 mg/ml of GLP-2, preferably about 5 to about 40 mg/ml, more preferably about 7 to about 30 mg/ml, even more preferably about 10 to about 20 mg/ml, and most preferably about 20 mg/ml of GLP-2.

In an embodiment of the invention, a liquid formulation of h[Gly2]GLP-2 suitable for lyophilization comprises: (1) preferably about 7 to about 30 mg/ml, even more preferably about 10 to about 20 mg/ml, and most preferably about 20 mg/ml of h[Gly2]GLP-2; (2) about 2 to about 5% of mannitol, preferably about 2.5 to about 3.5%, most preferably about 3%; (3) about 0.5 to about 1% of an amino acid stabilizer, which is preferably L-histidine; and (4) a phosphate buffer in an amount capable of buffering the reconstituted product to a pH of about 6.9-7.9, and preferably a pH of about 7.3 to about 7.4.

The GLP-2 formulations of the present invention are preferably filled in individual vials to the desired volume and the vials are subjected to a lyophilization process. The lyophilization process includes a temperature cycling process that is carefully controlled to ensure that drying proceeds uniformly. The drying process is continued until there is less than about 5% of water, preferably less than about 2% of water, and more preferably no more than about 1% of water, in the GLP-2 formulation.

A lyophilization process suitable for the present invention involves a freezing step and a two-step drying process. In an exemplary freezing process: (1) the formulation vials are first cooled from ambient temperature to about -1°C at about $2^{\circ}\text{C}/\text{minute}$, and then held at about -1°C for about 15 minutes, (2) next the vials are cooled from about -1°C to about -40°C at about $2^{\circ}\text{C}/\text{minute}$, and then held at about -40°C for about 4 hours.

In an exemplary first drying cycle, the temperature is increased from about -40°C to about -20°C at about $2^{\circ}\text{C}/\text{minute}$, and then held at about -20°C for about 14 hours under a vacuum of about 150 mT with a condenser temperature of about -80°C . In an exemplary second drying cycle, the vials are warmed from about -20°C to about $+20^{\circ}\text{C}$ at about $2^{\circ}\text{C}/\text{minute}$, and then held at about $+20^{\circ}\text{C}$ for about 14 hours at a vacuum of about 150 mT

and a condenser temperature of about -80°C until there is less than about 5% of water, preferably less than about 2% of water, and more preferably no more than about 1% of water. The vials are then preferably stored at about 4°C .

The present invention also provides a medically useful kit comprising: (1) at least one vial containing the lyophilized freeze-dried GLP-2 formulation of the invention; (2) at least one vial of sterile water for reconstitution; (3) instructions directing reconstitution; and (4) optionally an injection device for administration. To use the kit, the user mixes the water with the formulation vial, preferably by transferring the water to the formulation vial. The lyophilized formulation of the present invention rapidly dissolves upon reconstitution and, when reconstituted, is stable for at least about 12 hours, preferably up to about 24 hours, at 4°C . In a preferred embodiment, reconstitution of the lyophilized formulation is carried out using sterile water, preferably no more than about 1 mL of sterile water per dose of GLP-2. To reconstitute, the sterile water may be drawn into a syringe and then transferred to the vial containing the lyophilized GLP-2 formulation.

The following examples are given to illustrate the present invention. It should be understood, however, that the invention is not to be limited to the specific conditions or details described in these examples. Throughout the specification, any and all references to a publicly available document, including a U.S. patent, are specifically incorporated by reference.

Example 1: Formulation and Lyophilization of h[Gly2]GLP-2

The purpose of this example was to prepare a lyophilized formulation of the GLP-2 peptide h[Gly2]GLP-2.

A base formulation buffer, comprising 35 mM sodium phosphate at pH 7.4, was prepared as follows: (1) purified water was added to a sterile, depyrogenated flask; (2) sodium heptahydrate was added to the flask; and (3) monobasic sodium phosphate monohydrate was added to the flask. The buffer was mixed and the pH was verified to be 7.4 ± 0.2 . The base formulation buffer was then used to dilute the GLP-2 peptide h[Gly2]GLP-2 liquid bulk drug substance to a concentration of 10 mg/mL. L-histidine was then added to a final concentration of 7.76 gm/L, and mannitol was added to a final concentration of 30 gm/L.

The preparation was carefully mixed, followed by filtering the preparation through a $0.22\text{ }\mu\text{m}$ filter into a sterile filling tank. The GLP-2 preparation was then aseptically filled, in

1 ml aliquots, from the tank into 3 cc sterile USP Type I glass vials, which were then partially capped with sterile rubber stoppers and placed into lyophilization trays.

The vials were then loaded into the lyophilizer, and the lyophilization cycle was commenced by pre-freezing the formulation to a temperature of $-40 \pm 2^{\circ}\text{C}$ for about 4 hours.

5 In the freezing step, the formulation vials were first cooled from ambient temperature to -1°C at $2^{\circ}\text{C}/\text{minute}$ and then held at -1°C for approximately 15 minutes. This first freezing step was followed by cooling the vials from -1°C to -40°C at $2^{\circ}\text{C}/\text{minute}$, and the vials were then maintained at -40°C for 4 hours.

10 In the first and primary drying cycle, the temperature was increased from -40°C to -20°C at $2^{\circ}\text{C}/\text{minute}$ and then held at -20°C for about 14 hours under a vacuum of 150 mT with a condenser temperature of -80°C . In the second drying cycle, the vials were warmed from -20°C to $+20^{\circ}\text{C}$ at $2^{\circ}\text{C}/\text{minute}$ and then held at $+20^{\circ}\text{C}$ for about 14 hours at a vacuum of 150 mT and a condenser temperature of -80°C . The second drying cycle was continued until there is less than about 5% of water, preferably less than about 2% of water, and more preferably no more than about 1% of water, remaining in the GLP-2 formulation. The vials were then stored at 4°C .

At the end of the lyophilization cycle, the vials were purged with filtered nitrogen and the rubber stoppers were fully depressed into the vials. The stoppered vials were removed from the lyophilizer and permanently sealed with a crimped aluminum seal and capped with a polypropylene flip-off button.

Example 2: Screening of Amino Acid to Stabilize the Formulation

The purpose of this example was to determine the effect of various amino acid additives on the stability of GLP-2 following exposure to elevated temperatures.

25 The h[Gly2]GLP-2 formulation was tested with several amino acids as set out below. The tested formulations comprised: (1) h[Gly2]GLP-2 at a concentration of 10 mg/ml; and (2) the additives listed below. The pH of the composition was maintained between 7.1-7.5.

1. 10 mM phosphate, 10 mM Glutamate
- 30 2. 10 mM phosphate, 10 mM Glutamate, 50 mM Arginine
3. 10 mM phosphate, 10 mM Citrate
4. 10 mM phosphate, 10 mM Citrate, 50 mM Arginine
5. 10 mM phosphate, 100 mM Citrate
6. 10 mM phosphate, 100 mM Citrate, 50 mM Arginine

7. 10 mM phosphate, 10 mM Serine
8. 10 mM phosphate, 10 mM Serine, 50 mM Arginine
9. 10 mM phosphate, 10 mM Proline
10. 10 mM phosphate, 10 mM Proline, 50 mM Arginine
11. 10 mM phosphate, 10 mM Histidine
12. 10 mM phosphate, 10 mM Histidine, 50 mM Arginine
13. 10 mM phosphate, 10 mM Glycine
14. 10 mM phosphate, 10 mM Glycine, 50 mM Arginine
15. 10 mM His, 10 mM Glycine
16. 10 mM His, 10 mM Glycine, 50 mM Arginine

Following preparation, the samples were lyophilized according to the protocol of Example 1, stored at 40 °C for 14 days, diluted to 0.4 mg/ml, and then heated at 60 °C for 4 hours.

All of the formulations containing arginine precipitated upon heating (Formulations 2, 4, 6, 8, 10, 12, 14, and 16). Formulation 5 (100 mM citrate) and Formulation 15 (L-histidine and glycine) also precipitated. Formulations comprising L-histidine, 10 mM citrate, serine, proline, glutamate, and glycine (Formulations 1, 3, 7, 9, 11, and 13) showed similar stability when these compounds were used without the addition of other amino acids. (See Figure 1.)

As shown in Figure 2, when L-histidine was used as a stabilizer in combination with a phosphate buffer, the GLP-2 peptide remained stable following heat stress for 4 hours at 60 °C.

Example 3: Screening Bulk Agents

The purpose of this example was to determine the effect of various bulk agent additives on the stability of a GLP-2 peptide following exposure to elevated temperatures.

The following formulations of the GLP-2 peptide h[Gly2]GLP-2, at a concentration of 0.4 mg/ml, were lyophilized according to lyophilization process of Example 1. The compositions were then reconstituted and heated to 60 °C.

1. 25 mM histidine, 35 mM phosphate, 3% mannitol
2. 50 mM histidine, 35 mM phosphate, 3% mannitol
3. 75 mM histidine, 35 mM phosphate, 3% mannitol
4. 25 mM histidine, 25 mM phosphate, 3% sucrose

5. 25 mM histidine, 25 mM phosphate, 3% trehalose
6. 25 mM histidine, 25 mM phosphate, 3% maltose
7. 25 mM histidine, 25 mM phosphate, 3% lactose

As shown in Figures 3 and 4, the reverse phase HPLC data (Fig. 3) demonstrate that the mannitol samples (Formulations 1, 2, and 3) exhibited the least amount of GLP-2 degradation. In addition, all three L-histidine concentrations (25 mM, 50 mM, and 75 mM) showed comparable stability. The SE-HPLC analysis (Fig. 4) also showed that, except for maltose and lactose (Formulations 6 and 7), the GLP-2 analog in all of the formulations eluted as a single peak without aggregation. Formulations 6 and 7 gave an additional high molecular weight (HMW) impurity peak that accounted for approximately 6%. However when these samples were heat stressed at 60 °C, the high molecular weight impurity aggregates increased to approximately 20% in Formulations 6 and 7.

Accordingly, mannitol and sucrose were determined to be acceptable candidates for addition to the GLP-2 formulations of the invention.

Example 4: Screening Bulk Agents

The purpose of this example was to compare the effectiveness of the bulk agent additives sucrose and mannitol on the stability of GLP-2 following exposure to elevated temperatures.

The following formulations of h[Gly2]GLP-2, at 10 mg/ml, were prepared and the stability of GLP-2 in each formulation was analyzed. The concentration of sucrose in Formulation 2 was increased to 5% to satisfy physiological osmolarity.

1. 35 mM phosphate, 50 mM histidine, 3% mannitol, pH 7.4
2. 35 mM phosphate, 50 mM histidine, 5% sucrose, pH 7.4
3. 35 mM phosphate, 25 mM lysine, 3% mannitol, pH 7.4
4. 35 mM phosphate, 25 mM lysine, 5% mannitol, pH 7.4

The formulations were then lyophilized according to lyophilization process of Example 1, followed by reconstitution, and stability testing. The formulations were then heated to 60 °C for 4 hours, followed by stability testing.

All of the lyophilized samples stored at room temperature and at 40 °C remained stable.

The stability of the formulations following lyophilization and exposure to elevated temperatures was then measured. Formulation 1, comprising L-histidine and mannitol, did

not show evidence of GLP-2 degradation. However, Formulations 2, 3, and 4, comprising histidine/sucrose, lysine/mannitol, and lysine/mannitol, respectively, showed evidence of GLP-2 degradation over time (see Figure 6).

These results suggest that the addition of sucrose and lysine destabilizes the GLP-2 peptide (see also Figure 5), following exposure to elevated temperatures.

Example 5: The purity and quantity of h[Gly2]GLP-2

The purity of the GLP-2 is a measurement of peptide degradation or lack thereof. The quantity of GLP-2 is a measurement of the total content of the GLP-2 and hence it is an indication as to the quantitative amounts of peptide degradation, precipitation and/or aggregation.

To determine the purity and quantity of h[Gly2]GLP-2 reverse-phase HPLC is employed. Reverse phase chromatography is a bonded phase chromatographic technique that allows separation of compounds on the basis of their polarity. h[Gly2]GLP-2 is adsorbed onto the hydrophobic silica-based bonded reverse phase packing material of the column and is eluted as a single peak by increasing the hydrophobicity of the mobile phase with an acetonitrile gradient. The h[Gly2]GLP-2 sample is quantitated against a reference standard.

Equipment

Waters HPLC system or equivalent

Vydac (Hesperia, CA), C18 reverse-phase analytical column, 4.6mm x 25 cm, 5µm particle size, 300 Å pore size, or equivalent

Vydac (Hesperia, CA), C18 analytical guard cartridge, 4.6 x 30 mm, 5µm particle size, 300 Å pore size, or equivalent

Hamilton Digital Syringe or equivalent

Pipettes

Materials

Membrane filters (0.45µm)

HPLC standard glass vials, polypropylene inserts, and PTFE septa

Acetonitrile, HPLC grade

Milli-Q water

Trifluoroacetic acid (TFA), spectro grade

Ammonium bicarbonate, ACS grade

1M ammonium hydroxide

Procedure

Chromatographic conditions:

5

Mobile phase: Eluent A: 0.1% (v/v) TFA in Milli-Q water

Eluent B: 0.1% (v/v) TFA in acetonitrile

Autosampler: 2-8°C

Detector: wavelength set at 214 nm and sensitivity at 2 AU

10

Run time: 45 minutes

006227 2205160

Gradient conditions:

Time (minutes)	Flow Rate (mL/min)	% Eluent B	Curve Shape
0	1.0	30	6
1	1.0	30	6
30	1.0	60	6
35	1.0	30	6
45	1.0	30	6

Store column in 20% acetonitrile after use.

Preparation of 10 mM Ammonium Bicarbonate, pH 8 buffer:

Dissolve 0.20 gram of ammonium bicarbonate in approximately 200 mL of Milli-Q water. Adjust the pH to 8.0 ± 0.1 using 1 M ammonium hydroxide. Add Milli-Q water to final volume of 250 mL. Set expiration date of one week and store at 2-8°C. Allow buffer to warm to room temperature, then check pH and filter buffer through 0.45µm filter prior to use.

Preparation of standard:

Reconstitute h[Gly2]GLP-2 reference standard with filtered 10 mM ammonium bicarbonate, pH 8 buffer, to a concentration of 200 µg/mL.

Preparation of sample:

Reconstitute/dilute h[Gly2]GLP-2 test sample(s) in the same buffer used for the standard, to a concentration of 200 µg/mL. Prepare duplicate samples.

Analysis:

Inject 50 µL of standard solution 6 times, the % RSD of h[Gly2]GLP-2 peak retention time and area is not more than (NMT) 5%, the USP tailing factor of the h[Gly2]GLP-2 peak is between 1-2.

Inject 50 µL of blank (filtered 10mM ammonium bicarbonate, pH 8 buffer) once.

Inject 50 µL of h[Gly2]GLP-2 test sample once.

Inject 50 µL of standard solution once after ten injections of test sample and at the end of the run.

Data Processing and Calculations

Data Processing

Set the software provided with the HPLC system to integrate the area under every peak observed between 5 and 40 minutes, not including any peaks that correspond to those observed in the chromatogram of the blank injection.

Calculations

$$\% \text{ Purity} = \frac{\text{h[Gly2]GLP-2 peak area} \times 100}{\text{area of all peaks detected}}$$

$$\text{Concentration} = \frac{(\text{h[Gly2]GLP-2 peak area of sample} \times \text{conc. of standard}) \times \text{Dilution Factor (DF)}}{\text{ave. h[Gly2]GLP-2 peak area of standard}}$$

Example 6

A lyophilized formulation of 9 mg/ml of h[Gly2]GLP-2 was prepared in accordance with the method of example 1. This sample was tested for stability by measuring the purity and drug content of the sample at 4 °C and 25 °C using the method of Example 4. The results are presented in Table 1 and Table 2. As shown in the tables, the sample exhibited stability for at least 6 months and 18 months at 4°C and 25 °C, respectively.

Table 1: Storage Condition: 4°C

TEST METHOD	RESULTS					
	Time 0	3 Months	6 Months	9 Months	12 Months	18 Months
pH	7.4	7.4	7.2	7.0	7.4	7.4
Purity by RP-HPLC	99.3%	99.5%	99.3%	99.1%	99.0%	99.4%
Content by RP-HPLC (mg/ml)	9.0	8.7	8.9	8.7	8.7	8.8
Water Content or Residual Moisture (w/w)	1.0%	1.0%	1.0%	1.0%	1.0%	1.2%

006227 22005160

Table 2: Storage Condition: 25°C

TEST METHOD	RESULTS						
	Time 0 (release)	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
pH	7.4	7.4	7.5	7.4	7.2	7.3	7.2
Purity by RP-HPLC	99.3%	99.5%	99.3%	99.6%	99.3%	99.3%	99.4%
Content by RP-HPLC	9.0	8.7	9.1	8.8	9.3	8.7	9.0
Water Content or Residual Moisture (w/w)	1.0%	1.2%	1.2%	1.2%	1.3%	2.0%	1.3%

It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.